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(54) Title: THE USE OF THIOREDOXIN IN THE TREATMENT OF MALIGNANTLY TRANSFORMED CELLS IN ANIMALS AND MAN

(57) Abstract

The use of thioredoxin in the treatment of B lymphocytic leukemia and certain other malignant diseases. The enzyme can be used either alone or in combination with co-factors such as anti-immunoglobulins, interferons or interleukin 1,2,3, or 4.

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The use of thioredoxin in the treatment of malignantly transformed cells in animals and man.

Field of the invention

The present invention relates to a novel strategy for the treatment of B lymphocyte leukemias and certain other malignant diseases, including a method for potentiating the reactivity of lymphocytes responsive towards cancer cells expressing surface structures recognized by the patient's own cytotoxic cells. Examples of such cancers are malignant melanomas and colon cancer.

It is known from WO88/06891 that B-cell growth factors, and antibodies that mimic these, can be used for the induction of differentiation in certain malignant disorders. We describe here the use of an enzyme belonging to the thioredoxin family, such as MP6 cell line derived thioredoxin (MP6/Trx) for such induction of differentiation. The said enzyme will be used either alone or in combination with co-factors.

General outline of the invention and introduction

Cancer cells are characterized by uncontrolled growth. For some time there has been a concept that growth can be suppressed by inducing these cells to differentiate into a non proliferative state. Clinical trials have also been done in different leukemias with differentiation-inducing agents such as vitamins and interferons. However, no such trials have been done with more specific growth and differentiation factors, or antibodies, which only react with defined receptor structures. The present invention proposes to use such specific factors for cancer treatment, either alone or in combination with supporting, agents.

The development of normal cells into cancer cells is a multi-step process. During malignant transformation some cell types, for example some B lymphocytes (reference 1), acquire the ability to express receptors for defined growth factors and respond to these by proliferation or maturation. The tumor cells are thus "frozen" at a specific differentiation stage, characterized by a specific set of surface receptors. This block is, however, not irreversible. We here present a method for the use of an enzyme belonging to the thioredoxin family, and analogues to thioredoxin containing the same active site Cys-Gly-Pro-Cys, including monoclonal antibodies binding to the target structure, to be used alone or in common with co-factors, for the induction of terminally differentiated cells (end cells) which do not further divide. The said enzyme and co-factors are described. The strategy of clinical treatment is exemplified with B-cell chronic lymphocytic leukemias (B-CLL), which were induced to further differentiation (to a more mature stage) signified by impaired capacity to proliferate and the expression of a plasmacytoid morphology, as judged by surface markers, cytoplasmic immunoglobulin, and endoplasmatic reticulum.

For a resting B-cell, the initial activation signal, elicited by the antigen - immunoglobulin (Ig) interaction, must be followed by a series of finely tuned receptor-ligand signals and cell-cell interactions with other immunocompetent cells (1), to allow terminal plasma cell maturation. Several ligands for receptors involved in the transmission of growth and differentiation controlling signals in human B cells have been defined and the genes cloned. These include interleukin 1 (IL-1) to interleukin 6 (IL-6), low molecular weight B cell growth factor (LMW-BCGF), sCD23, lymphotoxin (LT), tumor necrosis factor (TNF), interferon-γ (IFN-γ) (1,2).

To grasp the concept of differentiation therapy it is important to understand how normal cells develop. In the

bone marrow, different functionally specialized cell types develop as a result of differentiation (commitment) of the multipotent stem cells. This differentiation gives rise to precursors of various cell linages (B-cell linage, T-cell linage, myeloid linage). Subsequent phenotypic changes of such unipotent cells into end cells is called maturation or terminal differentiation. The activation of human B-cells from a resting stage, leading into further differentiation and maturation and the terminal stage proceeds through at least two steps.

1) The activation step, where the cells are exposed to activating factors. For the B-cell series these are: Antigens; anti-immunoglobulins (anti-idiotypes); interleukin 1, 2 and 3 and sub-components thereof, interleukin 4 (IL4) and antibodies to the IL4-receptor; reagents acting on the C3d-receptor (CD11c), such as polymerized complement 3d or antibodies to the C3d receptor (anti-gp140); anti-gp35 (CD20). Phorbol esters, such as TPA or PMA are used experimentally in vitro as potent competence-inducing agents, but these can however only serve as models since they are toxic and incompatible with clinical use. The phorbol esters act on protein kinase C (PKC) and in their function mimic biologically active agents. Other experimental competence-inducing agents of importance are: solid phase protein-A; inactivated Staphylococcus Aureus Cowan I (SAC); Poke-weed Mitogen (PWM); non-transforming or inactivated Epstein-Barr Virus (EBV) (from the non-transforming strain P3HR1 or UV-inactivated virus) lipopolysaccharides (LPS).

2) The progression step. The activation step induces receptors for various progression signals such as : IL-2; B-cell growth factor II or TRF, now called IL5; low molecular weight BCGF (12K BCGF); Namalwa-derived 60K BCGF; antibodies to CD23 (a p45 protein expressed on the B-cell surface of IgM+, IgD+ cells, FcE receptor 2 (FcER2) antibodies to CD40, a p50 antigen present mainly on B-

cells and on urinary bladder carcinoma cells, but also on cervical and lung carcinoma cells, furthermore IL-6 (previously called B-cell differentiation factor (BCDF). The following list is a brief explanation of abbreviations used in the present specification.

BCDF: B-cell differentiation factor
BCGF: B-cell growth factor
B-CLL: B-cell chronic lymphocytic leukemia
BSF: B-cell stimulating factor
C3d: Sub-component of complement factor C3
CD23: A p45 protein expressed on cells of the B-lymphocyte linage,
CD40: A p50 protein expressed on B-cells and on bladder carcinoma cells
EBV: Epstein-Barr virus
gp35: Glycoprotein 35K molecular weight, belonging to the CD20 group (cluster of differentiation group)
gp140: Glycoprotein 140K molecular weight, with C3d-receptor function
IgD: Immunoglobulin class D
IgM: Immunoglobulin class M
IL-1, IL-2, IL-3, IL-4, IL-5: Interleukin 1, 2, 3, 4, 5
LPS: Lipopolysaccharides
Molt4: A T-lymphoma derived cell line
p45: A 45K molecular weight membrane protein
PMA: 4-phorbol 12-myristate 13-acetate
PWM: Poke weed mitogen
SAC: Staphylococcus aureus Cowan I
Solid phase protein-A: Matrix (Sepharose for example) - bound protein-A
TPA: Tumor promoting agent
TRF: T-cell replacing factor
T-T hybridoma: A somatic cell hybrid between two different T-cells.

TNF Tumor necrosis factor
MP6/Trx: MP6 T-T hybridoma cell line produced enzyme of thioredoxin family.

Detailed description of the invention

A 12 kDa B cell stimulatory factor (BSF) secreted by a human $CD4^+$ T cell hybridoma (MP6), was previously shown to facilitate growth of normal and malignant human B lymphocytes.
We have now purified this lymphokine and identified it as a member of the human thioredoxin family and named it MP6/Tr: Thioredoxin is a well-characterized enzyme catalyzing thiol-disulphide interchange reactions and net protein disulphide reductions via a Cys-Gly-Pro-Cys active site. We used normal peripheral blood or tonsillar B lymphocytes as target cells for monitoring biological activity . But monoclonal B cells, B-lymphoblastoid cell lines, or B cells derived from B-type of chronic lymphocytic leukemia (B-CLL), were target cells par excellence, since they required MP6/Trx for cytokine induced proliferation and differentiation in vitro, when tested under suboptimal cell culture conditions.
Pre-activated cells did proliferate in response to the recombinant or natural ligands: interleukin 2 (IL-2), interleukin 4 (IL-4), low molecular weight BCGF (LMW-BCGF), tumor necrosis factor-α, (TNF-α), or anti-CD40, only if MP6/Trx was added. Antibodies to thioredoxin blocked the effect. These results assign an important role to extracellular thioredoxin in the regulatory events involved in receptor-ligand interactions and subsequent signal transduction in normal B-cell activation and in B-CLL leukemogenesis.

The present invention relates to a novel method for the treatment of such malignantly transformed cells in mammals and in man, which are sensitive to the co-factors mentioned below and to thioredoxin. The method is characterized by the administration of a therapeutically adequate amount of thioredoxin. If necessary, said enzyme is administered following a period of pre-treatment with a co-factor capable of inducing the malignantly transformed cells to become sensitive to said enzyme. Example of such co-factors, are given in Table 1 below. It is foreseen that the administration of the enzyme thioredoxin can be made simultaneously with the co-factor.

The term "thioredoxin" as used in the present specification is understood to include the thioredoxin enzyme family and analogues of thioredoxin containing the active site Cys-Gly-Pro-Cys, specifically the MP6 cell line-derived thioredoxin.

More precisely, the novel method of treatment by the present invention can be applied to stem-cell disorders, hematopoetic malignancies, for example leukemias, B-cell leukemias and B-cell chronic lymphocytic leukemias, and other tumors which express co-factor receptors and respond to thioredoxin. For example, bladder carcinomas expressing the CD40 antigen can potentially be treated in the described fashion. Thioredoxin as well as the co-factors listed in Table 1 are substances which are known as such. They are, however, not in every instance known to have therapeutic utility.

The choice of a suitable co-factor is no critical parameter of the invention. There are experimental methods available which will enable the skilled worker to establish whether a specific co-factor as listed in Table 1 acts in synergy with the thioredoxin. It is, however, preferred to use IL-2 as co-factor. Also IL-4 and TNF-α may be mentioned as preferred co-factors.

The invention in another aspect relates to thioredoxin for use in the treatment of malignantly transformed cells in animals and in man, in particular for use in such malignantly transformed cells which are sensitive to thioredoxin. Also in this aspect, if necessary, thioredoxin is administered following a period of pre-treatment with a co-factor as described, which is capable of inducing the malignantly transformed cells to develop sensitivity for thioredoxin. Another aspect of the invention relates to the use of thioredoxin in the preparation of a medicament for treatment of malignancies. Such a medicament may comprise a co-factor as described above. Even though thioredoxin as well as co-factors as exemplified in Table 1 are known in the art, pharmaceutical preparations containing thioredoxin or of a combination of thioredoxin and a co-factor according to Table 1, are novel and represent as such an additional aspect of the present invention.

It is foreseen that such malignancies which are sensitive to treatment with IL-2, such as malignant melanomas, will be suitable targets for treatment with thioredoxin, suitably in combination with a co-factor.

It is also foreseen that cellular immunity (T-cells and NK-cells) can be strengthened by treatment with thioredoxin, optionally in combination with a co-factor as described.

In clinical practice, thioredoxin, co-factors or combinations thereof are administered in a manner which is analogous with known ways of administering medicaments for the treatment of cancer. Thus, administration will preferably be made by infusion or by intramuscular deposition.

The amount in which thioredoxin and/or co-factors is administered will vary within a wide range and will depend on various circumstances such as the severity of the disease and the age and the state of the patient. As an example of a suitable dosage interval can be mentioned a dosage which will provide a serum or plasma level of thioredoxin which is from about 2 to about 100 times the naturally occurring thioredoxin serum or plasma level.

The following Table 1 gives a list which exemplifies co-factors which may be used. The designation E indicates that the co-factor mainly is experimental and has possible use for diagnostic purposes. The designation C indicates that the co-factor has clinical use.

Table 1. Co-factors

E	Phorbol esters such as TPA
E	Antigens
C	Anti-Immunoglobulins (anti-idiotypes)
C	Interleukin 1 and sub-components thereof
C	Interleukin 2 and sub-components thereof
C	Interleukin 3 and sub-components thereof
C	Interleukin 4 (BSF1)
C	Anti-IL4-receptor antibodies
E	Poke weed mitogen
E	Lipopolysaccharides
E	Epstein Barr virus, non-transforming or inactivated
C	C3d receptor (CD11c) reactive agents C' and anti-receptor (gp 140) antibodies
C	Anti-gp35 (CD20)
E	SAC, Inactivated Staphylococcus aureus Cowan I
E	Solid-phase protein A
C	Interferons (alfa, beta and gamma)
C	Vitamins (in particular vitamin A, D, and biologically active derivatives
C	Leukotriene B4
C	TNF-α

The enzyme thioredoxin as used in the present invention is preferably of human origin. It is an enzyme catalyzing thiol-disulphide interchange reactions and net protein disulphide reductions via Cys-Gly-Pro-Cys active site. Human thioredoxin is preferably of human lymphocyte origin although other origins can be used. However, also the use of animal including mammal thioredoxin, procaryotic thioredoxin obtained e.g. from E. Coli and thioredoxins produced by genetically engineered expression vectors is included in the scope of the invention.

Thioredoxin, also known as thiol-oxidoreductase, is a ubiquitous 12 kDa protein with a redox-active disulphide (3); it is usually reduced by NADPH and the flavoprotein thioredoxin reductase. Reduced thioredoxin is a hydrogen donor for ribonucleotide reductase, an essential enzyme making deoxyribonucleotides for DNA synthesis. Thioredoxin is also involved in regulatory events (3), such as the light-dependent activation of photo-synthetic enzymes in the chloroplast of plant cells (4), and activation of glucocorticoid receptors to a steroid binding state (5). Thioredoxin regulates enzyme activity by thiol redox control which involves reduction of protein disulphides with a rate that is about 10^5 times faster than that of dithiotreitol (DTT) (3). Mammalian thioredoxins have been isolated and characterized (3,6). The distribution has been studied by immunohistochemical methods and thioredoxin was shown to be related to protein secretion and partly membrane associated (6). Recently a human thioredoxin gene was cloned by Wollman et al. (7). The gene was found to be expressed in activated, but not in resting lymphocytes. Originally the thioredoxin was reported to be an IL-1 like factor, derived from an Epstein-Barr virus containing B-cell line (7). Tagaya and co-workers (8) showed that the IL-2-receptor/Tac-inducing factor, also called adult T cell leukemia (HTLV-1) derived factor (ADF), was homologous to or identical with thioredoxin from analysis of a c-DNA clone.

The present invention assigns new biological functions for the thioredoxin family of enzymes and expands its role in lymphocyte activation.

Target cells in clinical situations:

Target cells in clinical situations are all such malignantly transformed cells that can respond to thioredoxin, especially MP6/Trx, by differentiation, including all those malignant cells that can be induced to express binding sites for thioredoxin and respond to this. Such induction can be exerted by the co-factors described in Table 1 or by other means.

Experimental evidence

The MP6 is a $CD4^+$ T helper cell hybridoma, previously isolated and cloned by us (9). The MP6 clone constitutively secretes as 12-14 kDa B cell stimulatory factor (BSF-MP6) inducing proliferation and IgM/IgG secretion in normal (9, 10), as well as in malignant pre-activated B cells of B-type chronic lymphocytic leukemia (B-CLL) (11). The IL-2 receptor expression was also enhanced by BSF-MP6 (12). Kishimoto and Honjo et al. have demonstrated that mRNA from MP6 cells did not hybridize with cDNA probes for IL-1α, IL-1β, IL-4, IL-5 nor IL-6 (12). Using various cellular assays, the MP6 supernatant was shown to lack activities of LMW-BCGF, TNF-α, and -β, IFN-α, -β, -γ, granulocyte-monocyte colony stimulating factors (GM-CSF), IL-1, IL-2, IL-4, IL-5 and IL-6 (9).

In the experiments represented in Figures 1A and 1B, we utilized monoclonal cells derived from a patient with B-CLL. This clone (I83) represents G_o arrested B-cells inducible to differentiation or differentiation accompanied by proliferation depending on the co-stimulatory signals (11) when activated by 12-O-Tetradecanoyl-phorbol 13-acetate (TPA) or by Staphylococcus aureus Cowan I (SAC). I83 cells were pre-activated by SAC for 2 days, to mimic antigen-triggered signals, or by a sub-optimal dose of TPA (1.6×10^{-7}M) for 1 h. The cells were refractory to any of the recombinant or natural B-lymphotropic lymphokines rIL-1β, rIL-2, rIL-4, rIL-6, rTNFα, LMW-BCGF, rIFN-γ, anti-CD40, or combinations thereof. SAC-activated cells did, however, respond to the lymphokines rIL-2, rTNFα, LMW-BCGF, when BSF-MP6, was added (Figure 1A). The signal pathway for SAC and TPA are different, in that TPA provides a non-physiological signal directly activating proteinkinase C, moving the cells into the cell cycle. Figure 1B illustrates that TPA activated cells responded to BSF-MP6 alone, and that the combinations of BSF-MP6 with several

different B lymphokines did not further increase DNA-synthesis. The exceptions are IL-4 and TNF-α which showed significant increase. This is in line with recent findings that TNF-α is an autocrine growth factor for human B cells (14), and we have previously shown in a series of experiments that IL-4 is strongly synergistic with BSF-MP6 for the induction of DNA-synthesis and for IgM secretion in TPA-activated cells (11).

A highly specific radioimmunoassay for human thioredoxin (13) (Figure 2), reveals that the BSF-MP6 factor is homologous to thioredoxin, or an analogue of thioredoxin as described earlier.

Serum-free medium of 24h conditioned medium MP6 contained 34 ng/ml of thioredoxin. Biological activity was monitored using the I83 B-CLL clone or normal tonsillar B cells and was confined to the 12 kDa region in gelfiltration experiments. Mammalian thioredoxins, after air oxidation, forms extra structural intra-molecular disulphide bonds leading to inactivation and aggregation (6). During the purification procedures and storage, preparations of BSF-MP6 were also easily oxidized, by atmospheric O_2, with a resulting loss of activity. When this fact was realized we started to perform the B-CLL activation experiments with a sub-optimal dose of 0.1 µM ß-mercaptoethanol present during the cultivation period. Higher concentrations of ß-mercaptoethanol (50-200 µM) promoted an increase in DNA-synthesis in itself, however. Growth stimulation of leukemic cells by thiols and disulphides in vitro is a well-known phenomenon (22). Figure 3 demonstrates the reconstitution of full biological activity in an 8 months old and inactive BSF-MP6 preparation after incubation with DTT. The observation that BSF-MP6 could be revived by reduction, is a typical feature of thioredoxins (6). The sample (serum-free, sterile, 24 h conditioned medium of MP6 stored at +4°C) was reduced for 30 minutes at 37°C

with 2 mM DTT prior to HPLC-gelfiltration. Almost all biological activity was recovered in the 12 kDa region.

The evidence from the radioimmunoassay and the reconstitution experiments that BSF-MP6 is homologous to thioredoxin or an analogue of thioredoxin as described earlier, prompted us to demonstrate whether thioredoxin, derived from another source could replace BSF-MP6 in the biological assay. Homogeneous human thioredoxin derived from placenta (13) was tested at a concentration of 0.5×10^{-7}M to 0.5×10^{-14}M, and showed biological activity down to 0.5×10^{-9} M.

The test results are given in Table 2 below.

The B-CLL cells were pre-treated with SAC 1:100 000 and IL 2 10 U/ml. 3H-Thy was added for the last 18h of a 72h incubation period. As is seen in Table 2, the thioredoxin was highly active.

Table 2. Stimulation of B-CLL cells by human placenta thioredoxin

Thioredoxin (M)	DNA-synthesis 3H-Thymidine incorporation (cpm)
0.5×10^{-7}	6327
control medium	1240

Biochemical characterization of this T-helper cell derived thioredoxin was performed by immunosorbent affinity chromatography, with Sepharose-coupled sheep anti-thioredoxin antibodies, combined with HPLC-gelfiltration. The procedure yielded highly purified thioredoxin as seen in Figure 4. The starting material was 24 h MP6 serum-free medium. The insert SDS-PAGE gel picture confirms the purity and molecular weight of the affinity purified material.

For the understanding of B cell differentiation, the clonal malignancy of B-CLL has proven to be a very useful model (11). The low proliferative capacity of B-CLL in vivo might, in part, be the result of a deficiency in growth factors produced by autologous non-B cells. BSF-MP6/thioredoxin is according to the evidence presented here one of the missing links. The evidence that BSF-MP6 with its thioredoxin activity, facilitates a proper response to the T-cell derived IL-2, IL-4, LMW-BCGF, and TNF-α, provides for the first time a possible explanation for the growth arrest of B-CLL cells. The notorious dysregulation of T helper lymphocytes in B-CLL patients (15) might result in a loss of thioredoxin production, necessary for the activation of the B-CLL cells, as shown by our results. Alternatively, the B-CLL cells themselves might be deficient for autocrine thioredoxin or require an initial dose of externally supplied thioredoxin to initiate its autocrine production.

Our present finding of identity between thioredoxin and a B cell stimulatory factor, strongly suggests a pivotal immunological role for this enzyme. It facilitates proper signal transduction and the well-known function of the enzyme to catalyze thiol-disulphide interchange reactions may (3) presumably allow dynamic three-dimensional correct receptor docking events to take place, although to gain knowledge about the exact mechanism further studies are required.

A useful in vitro model system for studies of B cell growth and differentiation controlling signals have been the human B-lymphotropic herpesvirus Epstein-Barr virus (EBV), since it induces proliferation and differentiation (16), by turning on genes obligatory for B cell growth (17). The B-CLL cells have, however, been refractory to attempts of EBV-transformation and one possible explanation to this resistance could be found in the fact that B-CLL cells, in addition to their low expression of

EBV-receptors (CD21), might be defective in their thioredoxin gen expression as indicated in this report and by our preliminary immunofluorescence analysis. Cellular thioredoxin, was recently suggested to be a principal hydrogen donor for herpes virus simplex-type 1 encoded ribonucleotide reductase (18). Thus, a lack of thioredoxin in the B-CLL cells could effectively block any herpesvirus multiplication in those cells.

Strategy of therapy

1) Thioredoxin by itself, especially MP6/Trx, should be administered when malignant cells already express any of the binding sites for thioredoxin.

2) Thioredoxin plus compounds in Table 1 should be administered in combination when the malignant cells do not express any of the binding sites for thioredoxin. This includes any of the specific compounds listed.

Figure Legends
Figure 1A and 1B

MP6 induces signals for DNA synthesis.
Cells derived from the B-CLL clone I83, kept frozen in liquid nitrogen, were revived and induced to DNA synthesis (Figure 1A and 1B) and immunoglobulin secretion (data not shown) with either SAC (Figure 1A) or TPA (Figure 1B) as activating signals. To induce proliferation and differentiation with SAC, cells were incubated with fixed bacteria for 2 days and then exposed to 100 U/ml of recombinant interleukins or natural B cell cytokines with or without 25% BSF-MP6 (v/v). Cells were cultured in flat-bottomed 96-well plates as 0.2 ml cultures (4×10^5 cells/well) or 2-ml cultures (4×10^6/well) (Costar, Cambridge, MA) in RPMI 1640 medium (Flow Laboratiories, Ayshire, GB), supplemented with 10% newborn calf serum (Gibco, Glasgow, G.B.), 2mM L-glutamine, 50 µg/ml gentamycin, 100 IU/ml of penicillin and 100 µg/ml of streptomycin. The cells were cultured for 6 days at 37°C in 5% CO_2-in air atmosphere. DNA synthesis was measured, assaying the incorporation of 1 µCi (=37kBq) per well of tritiated thymidine ([^{3}H]dThd; spec.act. 6.7 Ci/mmol; Dupont Scandinavia, Stockholm, Sweden), during the last 20-24 h of the cultivation period.
Heat-inactivated, formalin-fixed SAC-particles were used at a final concentration of 0.1%; TPA (Sigma Chemical Co., St. Louis, MO) was used at 1.6×10^{-7}M concentration; BSF-MP6 was obtained from serum-free 24 h cultures of the MP6 T cell hybridoma grown in Iscoves medium supplemented with 400 µg/ml of BSA (Boerhinger-Mannheim, Mannheim, W.Germany), 12.5 µg/ml of human transferrin (Kabi, Stockholm, Sweden), 50 µM ß-mercaptoethanol, 2mM L-glutamin, penicillin/streptomycin, concentrated on an Amicon device with an YM2 filter. rIL-2 was purchased from Amgen (Amersham, Amersham, G.B.). rIL-1ß (Genzyme, Boston, MA) had a specific activity of 10^8 U/mg and was used at 10

U/ml. rIL-4 was purchased from Genzyme (Boston, MA) and used at a final concentration of 100U/ml. rIL6 was a gift from Dr. Kishimoto, Osaka, Japan, and was used at 100 U/ml. Recombinant TNFα with a specific activity of 6×10^7 U/mg was used at 100 ng/ml and was a gift from Dr. G.R. Adolf, Ehrnst Boehringer Institute, (Wienna, Austria). LMW-BCGF was purchased from Cellular Products (Buffalo, NY) was used at a concentration of 10% v/v. Monoclonal anti-CD40 (G28-5 Mab) used at a concentration of 1 µg/ml was a gift from Dr. E Clark (Seattle, WA). rIFN-γ was obtained from Genentech (San Fransisco, CA). It had a specific activity of 3×10^7 U/mg and was used at 500 U/ml.

Figure 2

Radioimmunoassay for thioredoxin shows identity between BSF-MP6 and thioredoxin.

Solid line indicates pure human placenta thioredoxin. Broken line indicates BSF-MP6.
The radioimmunoassay was performed as described previously (13), briefly: 0.1 ml (0.2 pmol) of ^{125}I-labeled human placenta thioredoxin was incubated with 0.1 ml of standard human thioredoxin or unkonwn sample (MP6 supernatant concentrated 50-fold by ammonium sulphate precipitation), serialy diluted, and 0.1 ml (5 µg) of the IgG fraction of a rabbit antiserum against human thioredoxin, at 37° C with shaking for 4 hours. At the end of the incubation period 0.1 ml of a 1:5 diluted sheep anti-rabbit IgG antiserum was added and incubation was continued for 16 h at 4°C. The bound radioactivity (B) was separated from the free (F) by centrifugation for 30 min at 10 000 x g, followed by careful removal of the supernatant. The radioactivity was measured in both the pellet and the supernatant fractions using a LKB gamma counter (Bromma, Sweden). The ratio B/F was calculated and plotted against various standard thioredoxin concentration. The reactions in the absence of competing human thioredoxin and the rabbit anti-human thioredoxin antibody were used as negative controls. The radioiodination of thioredoxin was performed according to the chloramin-T method (13). All dilutions and incubations were carried out in phosphate buffered saline (PBS) containing 1 mg/ml of BSA.

Figure 3.

Reconstitution of BSF-MP6 activity by reduction with DTT. The biological activity of BSF-MP6 could be recovered by reduction as shown above. The thioredoxin-expressing T-hybridoma clone MP6 was cultured for 24 hours in Iscoves medium containing 400 µg/ml of BSA, 12.5 µg/ml of human transferrin, 50 µM ß-mercaptoethanol, 100 µg/ml of streptavidin, and 100 U/ml of penicillin and 2nM of L-glutamin, but the biological activity of the supernatant was lost after two weeks of storage at +4°C. The chromatogram shows the protein profile measured at OD_{205nm} (full scale: A=0.5), of a 200 µl MP6 supernatant, pre-treated with 2 mM DTT, then separated on a Superose-12 FPLC gelfiltration column (Pharmacia, Uppsala, Sweden), equilibrated with sterile phosphate buffered saline, pH 7.2. The flow rate was 0.4 ml/min. Fractions of 2 ml were collected and monitored for biological activity on B-CLL cells or on normal tonsillar B cells by measuring [^{3}H-]thymidine incorporation, as indicated by the vertical bars in the chromatogram. The molecular weight markers indicated were bovine serum albumin (68K), and ribonuclease A (13.7K).

Figure 4.

Chromatogram on BSF-MP6 purified on anti-thioredoxin affinity column plus HPLC-gelfiltration.

HPLC-chromatgraphy was performed on material that was bound and eluted at pH 3.0 from a sheep anti-thioredoxin Sepharose-protein A column (1.5 x 6 cm), coupled as previously described (21). Fractions of 1 ml were collected. Equilibration buffer was PBS de-airated in He_2. The first peak at the 12 kDa contains the biological activity. The second peak is salt.
Insert of SDS-polyacrylamide gel:
An 8-25% gradient SDS-polyacrylamide minigel (Pharmacia Phast gel system) was used for analysis of the purity. The samples are from left to right: MP6 serum free supernatant, before the affinity column; Affinity purified BSF-MP6/Thioredoxin; Human placenta thioredoxin; Molecular weight markers (Pharmacia) from top to bottom: 92.5 kDa, 67 kDa, 45 kDa, 30.1 kDa, 20.1 kDa, 14.7 kDa.

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What we claim is:

1. A method for the treatment of malignantly transformed cells in animals and man, which comprises the administration of a therapeutically adequate amount of thioredoxin or an analogue of thioredoxin containing the active site Cys-Gly-Pro-Cys, especially MP6/Trx.

2. A method according to claim 1 wherein the origin of the thioredoxin is
- animal
- mammalian
- human
- procaryotic or
- recombinant.

3. A method according to claims 1 or 2 wherein the thioredoxin is of human lymphocyte or other human origin.

4. A method according to claims 1-3, characterized in that the malignantly transformed cells are pre-treated with a co-factor capable of inducing binding sites for thioredoxin.

5. A method according to claim 4, characterized in that the said co-factor is selected from

(a) anti-immunoglobulins (anti-idiotypes)
(b) interleukin 1 and sub-components thereof
(c) interleukin 2 and sub-components thereof
(d) interleukin 3 and sub-components thereof
(e) interleukin 4 (BSF1)
(f) anti-IL4-receptor antibodies
(g) C3d receptor (CD11c) reactive C'agents and anti-receptor (gp 140) antibodies
(h) anti-gp35 (CD20)
(i) interferons (alfa, beta and gamma)
(j) vitamins
(k) leukotriene B4

(1) TNF-α

6. A method according to claim 5 wherein the co-factor is interleukin -2.

7. A method according to any of claims 1-6 for the treatment of stem cell disorders.

8. A method according to any of claims 1-6 for the treatment of hematopoetic malignancies.

9. A method according to any of claims 1-6 for the treatment of B-cell leukemias.

10. A method according to any of claims 1-6 for the treatment of B-cell chronic lymphocytic leukemia.

11. A method according to any of claims 1-6 for the treatment of other tumors which express binding sites/ receptors for, and respond to, thioredoxin or analogues thereof as defined in claim 1.

12. Thioredoxin for use in the treatment of malignantly transformed cells in animals and man.

13. Thioredoxin for use in the treatment of the disorders mentioned in claims 7, 8, 9, 10, and 11.

14. Thioredoxin for use according to claims 12 and 13 in conjunction with a co-factor capable of inducing the malignantly transformed cells to express binding sites for thioredoxin.

15. Thioredoxin for use according to claim 14, in conjunction with a co-factor as listed under (a)-(l) in claim 5.

16. Thioredoxin for use in therapy, optionally in conjunction with a co-factor capable of inducing

malignantly transformed cells to express binding sites for the said thioredoxin.

17. A pharmaceutical composition, comprising thioredoxin or analogues of thioredoxin containing the active site Cys-Gly-Pro-Cys as active ingredient.

18. A pharmaceutical composition according to claim 17, comprising also a co-factor capable of inducing malignantly transformed cells to express binding sites for thioredoxin or an analogue as defined in claim 16.

19. A pharmaceutical composition according to claim 17 which also contains a co-factor as listed under (a)-(l) in claim 5.

20. A pharmaceutical composition according to claim 19 wherein the said co-factor is interleukin -2.

21. The use of thioredoxin in the preparation of a medicament for the treatment of malignantly transformed cells in animals and man.

22. The use of thioredoxin in the preparation of a medicament for the treatment of the disorders mentioned in claims 7, 8, 9, 10, and 11.

23. A method for potentiating the reactivity of lymphocytes responsive towards cancer cells expressing surface structures recognized by the patient's own cytotoxic cells, by administering thioredoxin or an analogue of thioredoxin containing the active site Cys-Gly-Pro-Cys, optionally in conjunction with a co-factor as listed under (a)-(l) in claim 5.

24. The MP6/Trx variant of thioredoxin for use as specified in claims 12-16.

25. A pharmaceutical composition according to claims 17-20, wherein the thioredoxin component is MP6/Trx.

26. The use according to claims 21 and 22 of the MP6/Trx variant of thioredoxin.

Abstract

The use of thioredoxin in the treatment of B lymphocytic leukemia and certain other malignant diseases.

Figure 1A

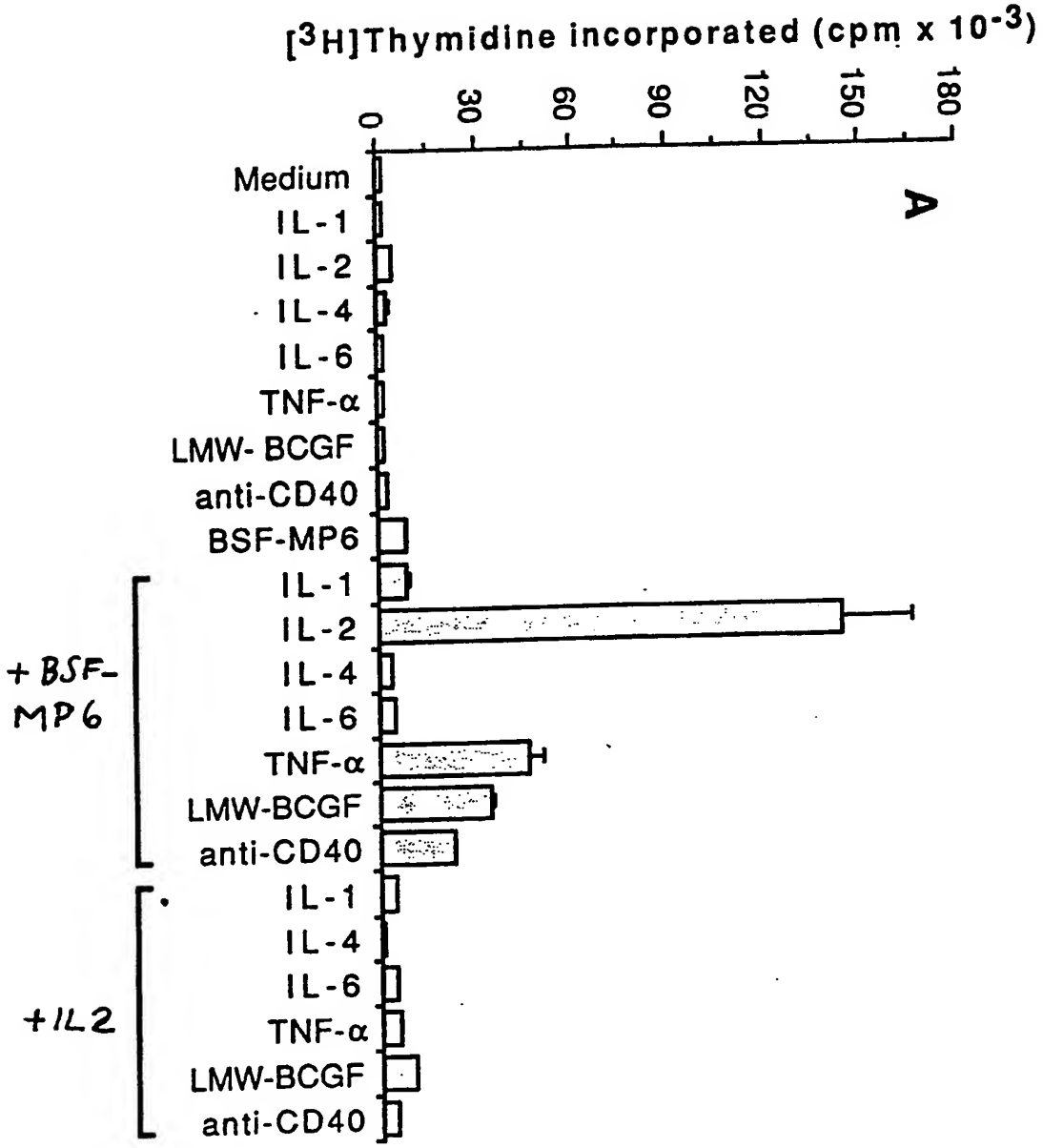
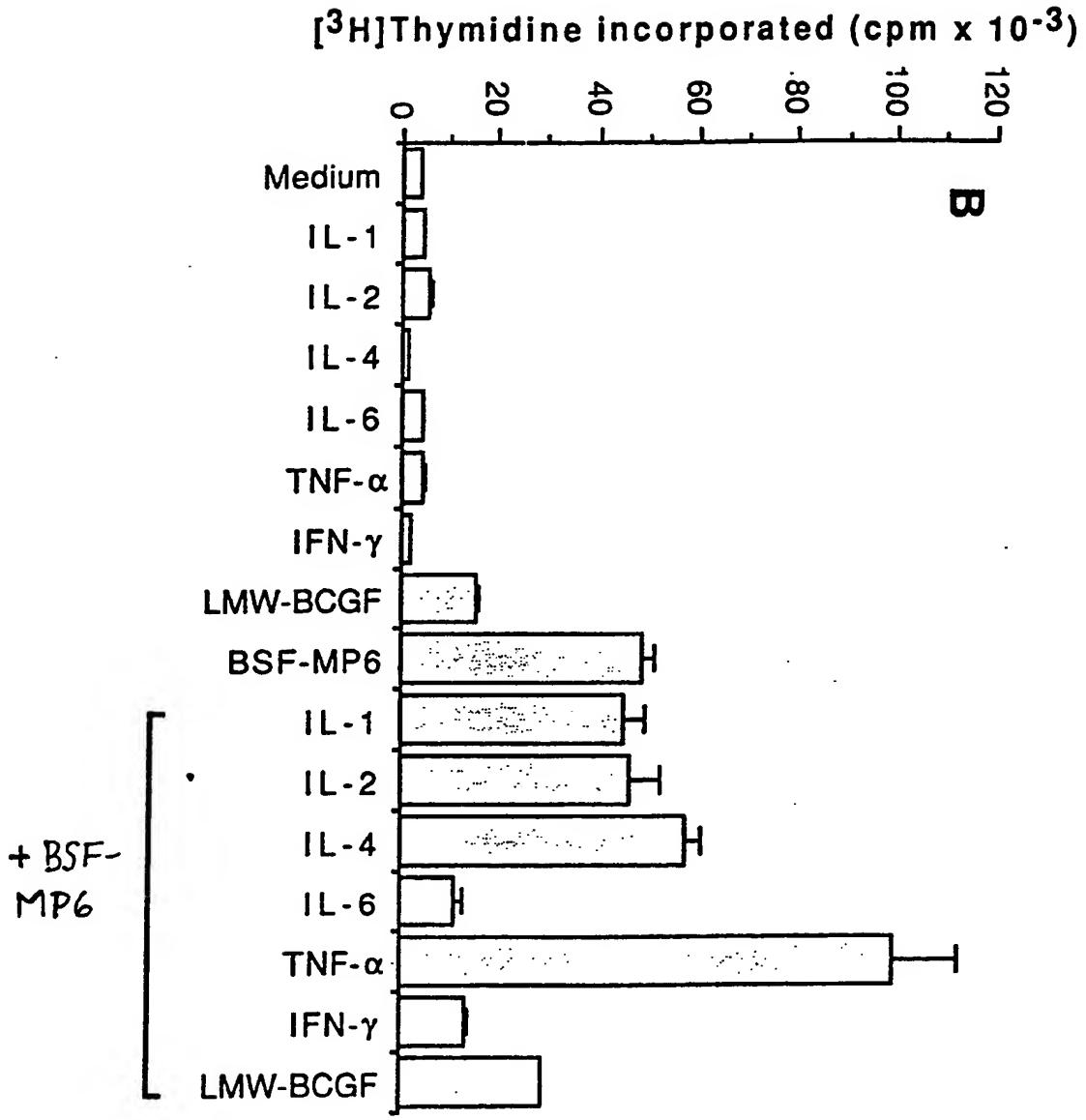

[3H]Thymidine incorporated (cpm x 10-3)
0
30
60
90
120
150
180
A
Medium
IL-1
IL-2
IL-4
IL-6
TNF-α
LMW- BCGF
anti-CD40
BSF-MP6
+BSF-MP6
IL-1
IL-2
IL-4
IL-6
TNF-α
LMW-BCGF
anti-CD40
+IL2
IL-1
IL-4
IL-6
TNF-α
LMW-BCGF
anti-CD40

Figure 1B


[3H]Thymidine incorporated (cpm x 10-3)
0
20
40
60
80
100
120
B
Medium
IL-1
IL-2
IL-4
IL-6
TNF-α
IFN-γ
LMW-BCGF
BSF-MP6
+ BSF-MP6
IL-1
IL-2
IL-4
IL-6
TNF-α
IFN-γ
LMW-BCGF

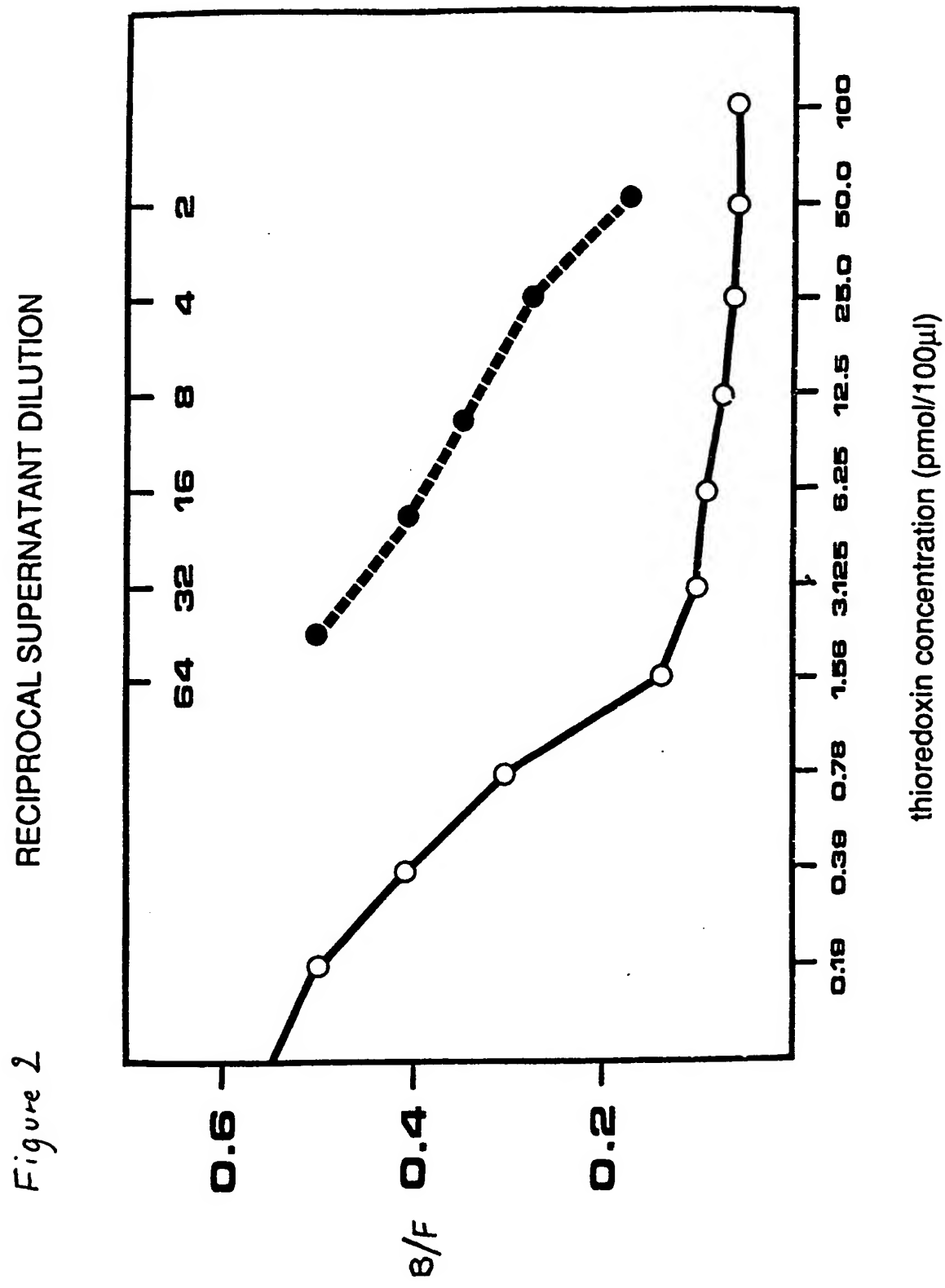
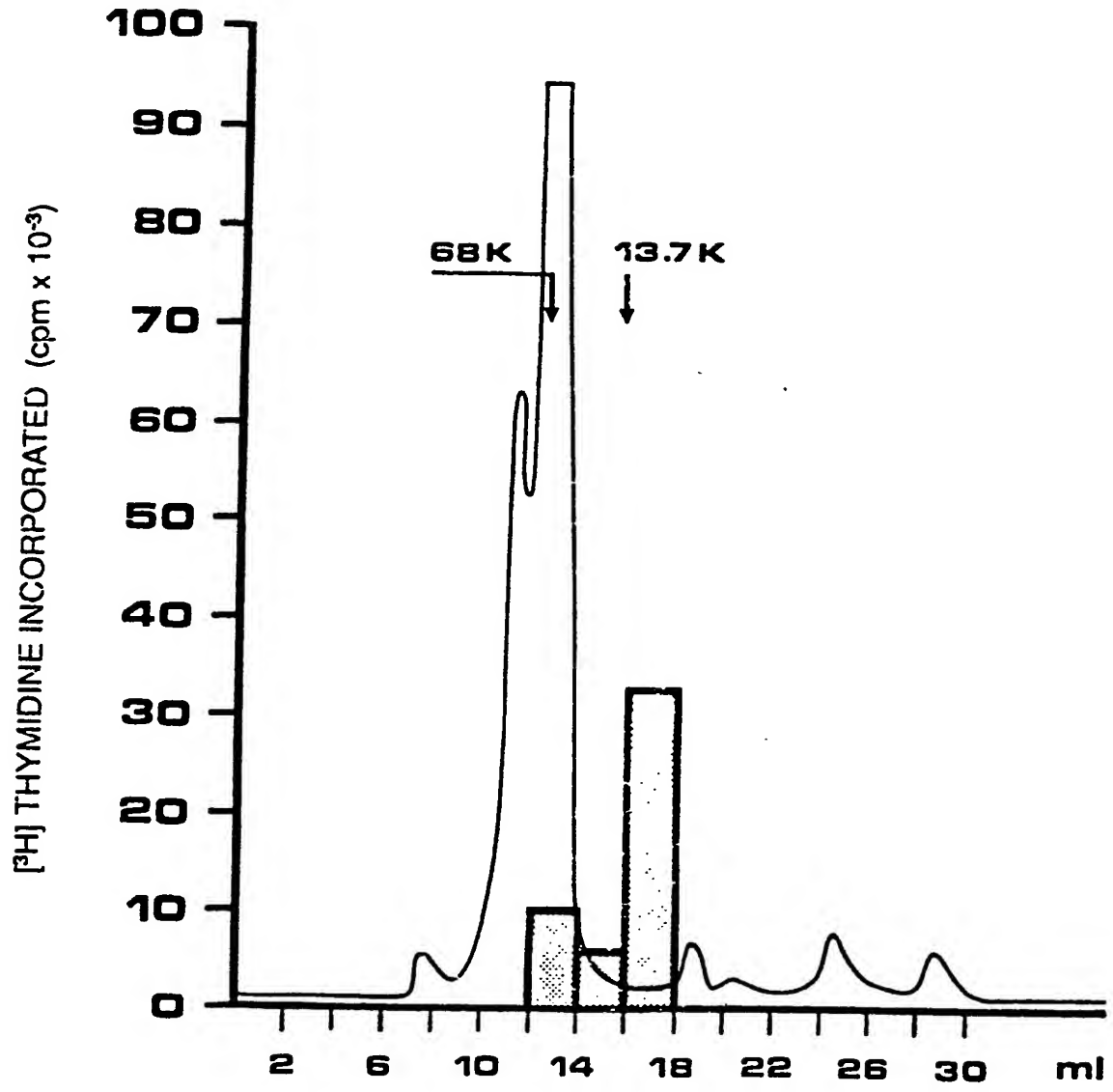

RECIPROCAL SUPERNATANT DILUTION
64 32 16 8 4 2
B/F
0.6 0.4 0.2
0.19 0.39 0.78 1.56 3.125 6.25 12.5 25.0 50.0 100
thioredoxin concentration (pmol/100µl)

Figure 2

Figure 3


100
90
80
70
60
50
40
30
20
10
0
[³H] THYMIDINE INCORPORATED (cpm x 10⁻³)
68K
13.7K
2
6
10
14
18
22
26
30
ml

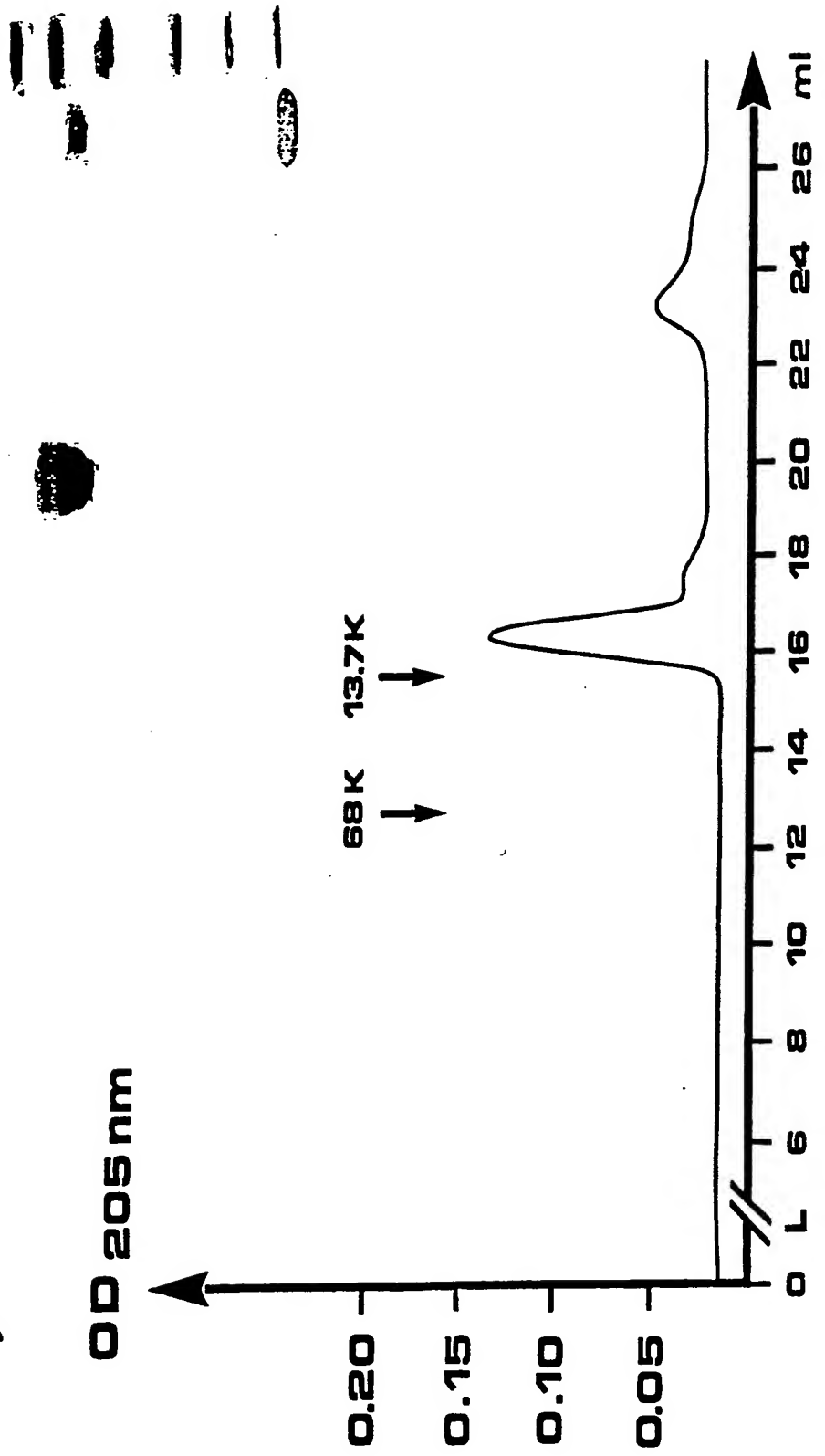

OD 205 nm
0.20
0.15
0.10
0.05
0 L 6 8 10 12 14 16 18 20 22 24 26 ml
68K
13.7K

Figure 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 90/00578

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 9/00, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A2, 0299206 (AJINOMOTO CO., INC.) 18 January 1989, see the whole document	1-3,6-14,17,21-22,24-26
Y		4-5,15-16,18-20,23
Y	WO, A1, 8806891 (AKTIEBOLAGET ASTRA) 22 September 1988, see the whole document	4-5,15-16,18-20,23
A	The Journal of Biological Chemistry, Vol. 263, No. 30, 1988 Emmanuelle E. Wollman et al: "Cloning and Expression of a cDNA for Human Thioredoxin ", see pages 15506-15512	1-26

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3rd December 1990	1990 -12- 07
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 111, no. 9, 28 August 1989, (Columbus, Ohio, US), Tagaya, Yutaka et al: "Production and activities of adult T-cell leukemia derived factor (ADF) ", see page 527, abstract 75509s, & Jikken Igaku 1989, 7(7), 764- 769 --	1-26
A	Chemical Abstracts, volume 110, no. 1, 2 January 1989, (Columbus, Ohio, US), Schallreuter, Karin U et al: "The activity and purification of membrane-associated thioredoxin from human metastatic melanotic melanoma ", see page 320, abstract 3440u, & Biochim.Biophys.Acta 1988, 967(1), 103- 109 --	1-26
A	Neumüller, Otto-Albrecht"Römpps Chemie-Lexikon", 1988, Franckh'sche Verlagshandlung, Stuttgart, see page 4248 "Thioredoxine" --	1-26

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 1-11, 23, because they relate to subject matter not required to be searched by this Authority, namely:

See Rule 39.1(iv)
Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 90/00578

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 90-11-01
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0299206	89-01-18	JP-A-	1085097	89-03-30
WO-A1- 8806891	88-09-22	AU-D-	1487888	88-10-10
		EP-A-	0305468	89-03-08
		JP-T-	1502592	89-09-07